Carbohydrate Research, 161 (1987) 127–132
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURAL STUDIES OF AN EXTRACELLULAR POLYSACCHARIDE (S-198) ELABORATED BY *Alcaligenes* ATCC 31853

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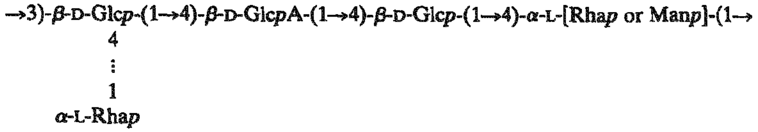
(Received August 5th, 1986; accepted for publication, September 22nd, 1986)

ABSTRACT

The structure of an extracellular polysaccharide, S-198, elaborated by *Alcaligenes* ATCC 31853 has been investigated; methylation analysis, specific degradations, and ^{1}H-n.m.r. spectroscopy were the main methods used. It is suggested that the polysaccharide is composed of "repeating units" with the structure



$$\begin{array}{l} \rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-[Rha}p\text{ or Man}p\text{]-}(1\rightarrow \\ \qquad\qquad 4 \\ \qquad\qquad \vdots \\ \qquad\qquad 1 \\ \qquad \alpha\text{-L-Rha}p \end{array}$$

A sugar residue in the chain may be either L-rhamnose or L-mannose and only ~50% of the residues contain the branching α-L-rhamnopyranosyl group. The polysaccharide further contains *O*-acyl groups. It belongs to a group of polysaccharides, elaborated by *Alcaligenes* and *Pseudomonas* species, which all have the same linear backbone (except that some of them do not contain L-mannose) without branching or with branches that differ in their chemical structures and/or positions.

INTRODUCTION

Several extracellular bacterial polysaccharides of considerable economic interest are composed of oligosaccharide repeating-units containing the structural element **1**.

$$\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow$$

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[*] On study leave from the Department of Chemistry, Dhaka University, Bangladesh.

0008-6215/87/$ 03.50

The simplest of these is gellan gum, elaborated by *Pseudomonas elodea.* It is composed of tetrasaccharide repeating-units with the structure **1**, and further contains *O*-acyl groups[1] that have recently been shown to comprise L-glycerate and acetate[2]. The deacetylated polysaccharide forms strong gels in the presence of cations. The other polysaccharides have branched structures and give highly viscous aqueous solutions that complicate structural studies. In S-130, or wellan gum, elaborated by an *Alcaligenes* species, an α-L-rhamnopyranosyl or α-L-mannopyranosyl group is linked to O-3 of the second D-glucopyranosyl residue in **1**[3]. S-88, elaborated by a *Pseudomonas* species, has almost the same structure, but with an α-L-rhamnopyranosyl end-group and an α-L-mannopyranosyl residue partially replacing the α-L-rhamnopyranosyl residue in the chain[4]. In S-194, or rhamsan gum, elaborated by an *Alcaligenes* species, a β-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl group is linked to O-6 of the first β-D-glucopyranosyl residue in **1**[5]. We now report structural studies of a polysaccharide, S-198, elaborated[6] by *Alcaligenes* ATCC 31853, which proved to be a fifth member of this group of polysaccharides.

RESULTS AND DISCUSSION

The polysaccharide, on acid hydrolysis, yielded L-rhamnose, L-mannose, and D-glucose in the relative proportions 31:15:51. It also contains D-glucuronic acid residues (~13%) and *O*-acyl groups that are mainly *O*-acetyl[6]. The absolute configurations of the sugars were determined as devised by Gerwig *et al.*[7].

The polysaccharide, before and after deacylation, gave highly viscous solutions and poor n.m.r. spectra. Better spectra were obtained using a polymeric product, prepared from the deacetylated polysaccharide by treatment with acid under mild conditions. The ^{1}H-n.m.r. spectrum of this material showed, *inter alia,* unresolved signals at δ 5.22 and 5.10 (1.3 H together), assigned to α-L-rhamnopyranosyl and α-L-mannopyranosyl residues, signals at δ 4.73 (H, $J_{1,2}$ ~7 Hz) and 4.53 (2 H, $J_{1,2}$ ~7 Hz), assigned to β-D-glucopyranosyl and β-D-glucopyranosyluronic acid residues, and at δ 1.31 and 1.26 (3.1 H together, $J_{5,6}$ ~6 Hz), assigned to H-6 of L-rhamnopyranosyl residues. In addition, some minor peaks at δ ~5.51 were observed.

Methylation analysis of the polysaccharide, without and with carboxyl-reduction of the methylated product, gave the sugars listed in Table I, columns *A* and *B*. The analyses show that the polysaccharide contains L-rhamnopyranosyl groups, and L-rhamnopyranosyl and L-mannopyranosyl residues linked through O-4. Evidence that these residues are pyranosidic and linked through O-4, and not furanosidic and linked through O-5, is given below. The polysaccharide further contains D-glucopyranosyl residues linked through O-3, through O-4, and through O-3 and O-4, and D-glucopyranosyluronic acid residues linked through O-4. It is difficult, from the relative proportions of these ethers, to construct a regular repeating-unit. It seems possible that the 4-substituted L-rhamnopyranosyl and L-mannopyranosyl residues may replace each other, as was observed[3] in S-88. The

TABLE I

METHYLATION ANALYSIS OF S-198 AND SOME DEGRADATION PRODUCTS[a]

Sugar[b]	T[c]	*Mole %*				
		A	B	C	D	E
1,2,3,5-Rhamnitol	0.13	—	—	6.6	2.8	—
1,2,3,5,6-Mannitol	0.38	—	—	13.6	8.6	—
2,3,4-Rha	0.48	10.8	16.4	—	—	36.6
2,3-Rha	0.94	14.9	9.3	—	5.9	7.6
2,3,4,6-Glc	1.00	—	—	31.9	12.6	41.5[d]
2,4,6-Glc	1.76	20.4	17.5	—	16.0	4.4
2,3,6-Man	1.92	22.8	16.2	—	14.8	10.0
2,3,6-Glc	2.16	20.2	16.0	26.2	28.3	—
2,6-Glc	3.08	10.9	9.0	—	—	—
2,3-Glc	4.0	—	15.6	21.7	11.2	—

[a] Key: *A*, methylated polysaccharide; *B*, methylated and carboxyl-reduced polysaccharide; *C*, methylated and carboxyl-reduced tetrasaccharide-alditol; *D*, methylated and carboxyl-reduced octasaccharide-alditol; *E*, product from uronic acid degradation. [b] 2,3,4-Rha = 2,3,4-tri-*O*-methyl-L-rhamnose, *etc.* [c] Retention time of the derived alditol acetate, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a DB-225 column at 190°. [d] Trideuteriomethyl at O-4.

low yields of 2,3,4-tri-*O*-methyl-L-rhamnose, 2,6-di-*O*-methyl-D-glucose, and 2,4,6-tri-*O*-methyl-D-glucose may further indicate that only some of the "repeating-units" contain a branch, linked to O-4 of a D-glucopyranosyl residue.

Controlled hydrolysis of deacylated S-198 with acid yielded a mixture of mono- and oligo-saccharides, from which a tetrasaccharide and an octasaccharide fraction were isolated. Methylation analysis of the alditols prepared from these fractions gave the sugars listed in Table I, columns C and D. The identifications of 1,2,3,5-tetra-*O*-methyl-L-rhamnitol and 1,2,3,5,6-penta-*O*-methyl-L-mannitol demonstrate that the corresponding sugars in S-198 are pyranosidic. These analyses are similar to those of the corresponding fractions obtained[3] from S-88, and the ^{1}H-n.m.r. spectra of the tetrasaccharide mixtures (Table II) and the octasaccharide-alditol mixtures (Table III) were similar. The oligosaccharides consequently have structures **2** and **3**, respectively, in which L-rhamnose and L-mannose may replace each other.

β-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-L-Sug

2

β-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-L-Sug*p*-(1→3)-β-D-
Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-L-Sug

3

Sug = Rha or Man

TABLE II

^{1}H-N.M.R. DATA FOR THE TETRASACCHARIDE MIXTURES OBTAINED FROM S-88 AND S-198

S-88			*S-198*		
δ (p.p.m.)	*J (Hz)*	*Integral (H)*	*δ (p.p.m.)*	*J (Hz)*	*Integral (H)*
1.34	~6	2	~1.32	6.4	1.5
1.36	~6				
4.54	7.8	1	~4.52	7.8	2
4.60	8.0	1			
4.73	8.0	1	4.71	8.1	1
4.86	1.2	0.1	4.84	1.2	0.4
4.89	1.2	0.3	4.87	1.0	
5.11	1.9	0.2	5.09	1.9	0.6
5.18	1.9	0.4	5.16	1.9	

The identifications of **2** and **3** demonstrates that S-198 and S-88 have the same linear backbone and differ only in the position and nature of the side chain.

The alditol acetates of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,4,6-tetra-*O*-methyl-L-mannose are difficult to separate by g.l.c. However, the corresponding acetylated aldononitriles are well separated, and, using these derivatives, it was demonstrated that the terminal sugar in **2** and **3** was D-glucose.

In order to locate the position of the side chain, fully methylated S-198 was treated with sodium methylsulfinylmethanide in dimethyl sulfoxide, followed by trideuteriomethylation. A hydrolysate of the product contained the sugars listed in Table I, column E. The course of the degradation is analogous to that reported[3] for S-88. The formation of 2,3,6-tri-*O*-methyl-4-*O*-trideuteriomethyl-D-glucose demonstrates that the *β*-D-glucopyranosyluronic acid residue in S-198 is linked to

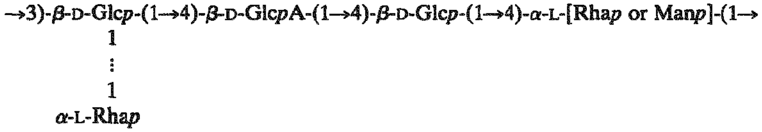
TABLE III

^{1}H-N.M.R. DATA FOR THE OCTASACCHARIDE-ALDITOL MIXTURES FROM S-88 AND S-198

S-88			*S-198*		
δ (p.p.m.)	*J (Hz)*	*Integral (H)*	*δ (p.p.m.)*	*J (Hz)*	*Integral (H)*
1.27	6.4	2.6	~1.28	6	2.7
1.31	6.3				
4.53	8.0	5	4.54	8	5
4.54	8.0		4.59	8	
4.56	8.0		4.61	8	
4.60	8.0				
4.62	8.0				
4.72	8.0	1	4.71	7.8	1
5.14	N.r.[a]	1	5.22	1.5	1
5.21	N.r.				

[a] Not recorded.

O-4 of a β-D-glucopyranosyl residue, which is linked only through this position. The disappearance of 2,6-di-*O*-methyl-D-glucose and the almost complete disappearance of 2,4,6-tri-*O*-methyl-D-glucose indicate that the sugar residues giving these ethers are linked to O-4 of the D-glucuronic acid residue, and are liberated as reducing sugar residues and degraded. As these sugars are linked through O-3, the sugars linked to this position, known to be L-rhamnose and L-mannose, should also be liberated as reducing sugars and degraded. A considerable decrease in the proportions of 2,3-di-*O*-methyl-L-rhamnose and 2,3,6-tri-*O*-methyl-L-mannose was also observed. However, the branch, which should be linked to O-4 of the branching D-glucopyranosyl residue, should survive. The only sugar which, in addition to tetra-*O*-methyl-D-glucose, was present in large proportion was 2,3,4-tri-*O*-methyl-L-rhamnose, indicating that the branches consist of single α-L-rhamnopyranosyl groups. The results further indicate that only part of the "repeating-units" contain this branch. The percentage of the units containing this branch is difficult to estimate, as the different analyses did not seem to be very accurate, but a value of ~50% is suggested. From the combined evidence, it is therefore suggested that S-198 is composed of "repeating-units" having structure **4**.



$$\begin{array}{l}\rightarrow3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow4)\text{-}\alpha\text{-L-[Rha}p\text{ or Man}p\text{]-}(1\rightarrow\\ \qquad\quad 1\\ \qquad\quad \vdots\\ \qquad\quad 1\\ \quad\alpha\text{-L-Rha}p\end{array}$$

4

In agreement with this structure, 2,3,4,6-tetra-*O*-methyl-D-glucose, about half of which had a trideuteriomethyl group at O-3 and the remainder had trideuteriomethyl groups at O-3 and O-4, was obtained on hydrolysis of fully methylated S-198 under mild conditions (during which mainly the α-L-rhamnosidic linkages should be cleaved), followed by trideuteriomethylation and complete hydrolysis of the product.

In order to eliminate other potential structures, with longer side-chains, S-198 was subjected to hydrolysis with acid under different conditions and the products were fractionated by ion-exchange and gel-permeation chromatography. Despite careful work-up, no neutral oligosaccharides were obtained and the acidic tetra- and octa-saccharides were not contaminated by higher, branched oligosaccharides. From these experiments, it therefore seems most unlikely that S-198 contained longer side-chains.

EXPERIMENTAL

The general methods and procedures for deacylation, partial hydrolysis, and uronic acid degradation were the same as in the investigation[4] of S-88.

Purification of S-198. — The polysaccharide (150 mg) was dissolved in water

(300 mL), and aqueous 1% cetyltrimethylammonium bromide (50 mL) was added with stirring. The precipitate was collected, dissolved in 4M aqueous sodium chloride (250 mL), and reprecipitated with ethanol. The precipitate was dissolved in water, the solution was dialysed against distilled water for 48 h, and the polysaccharide (138 mg) was recovered by freeze-drying.

*Characterisation of tetra-O-methyl-*D*-glucose.* — The mixture of methylated sugars was converted into the acetylated aldononitriles[8]. On chromatography on an SE-54 capillary column at 120→220° (2°/min), the retention times of the 2,3,4,6-tetra-*O*-methyl-D-glucose and -L-mannose derivatives were 5.5 and 5.9 min, respectively.

Partial hydrolysis of methylated S-198. — Fully methylated S-198 (5 mg) was dissolved in 1,4-dioxane–water (3:1, 10 mL), the solution was kept at 100°, and 2M trifluoroacetic acid was added to a concentration of 0.1M. The solution was kept at 100° for 4 h and then concentrated to dryness, and the product was reduced with sodium borohydride and remethylated, using trideuteriomethyl iodide. This material was hydrolysed to monomers, reduced with sodium borodeuteride, acetylated and analysed by g.l.c.–m.s. In the spectrum of the 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, ions at m/z 118 and 165, but not at 121 and 162, demonstrate that the whole material contained a trideuteriomethyl group at O-3. The presence of ions at m/z 45, 161, and 164, with the two latter ions of approximately equal intensities, demonstrates that ~50% of the material contained a trideuteriomethyl group at O-4. Other ions in the mass spectrum were also in agreement with this conclusion.

ACKNOWLEDGMENTS

This work was supported by a maintenance grant from the International Seminar in Chemistry, University of Uppsala (to T. Chowdhury) and a grant from the National Swedish Board for Technical Development. We thank Dr. Per-Erik Jansson for valuable discussions.

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